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Secondary structure and phylogeny of the chloroplast 23S rRNA gene from the brown alga *Pylaiella littoralis*

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Abstract

The entire nucleotide sequence of a 23S rRNA gene from the brown alga *Pylaiella littoralis* (L.) Kjellm has been determined. The predicted length of the 23S rRNA is 2948 nucleotides, including the 4.5S rRNA-like region at the 3′ end of the molecule. The putative transcript has been folded into a secondary structure by comparison to existing structure models, and the predicted helical regions were inspected by identifying compensatory downstream base changes. The 23S rRNA secondary structure presented here has features that are unique to *P. littoralis* (no other chromophyte or red algal 23S rRNA sequences are yet available), but has none of the features specific to the chloroplast rRNAs of green plants and green algae. The *Pylaiella* sequence was aligned with analogous plastidial and eubacterial gene sequences, and the alignment was used to construct a phylogenetic tree. The plastidial sequences formed a coherent cluster closely associated with the 23S rRNA of the cyanobacterium *Anacystis nidulans*. Within the plastid group, the *P. littoralis* sequence was most closely related to that of *Euglena gracilis* confirming earlier analyses based upon 16S rRNA sequences.

Introduction

Plant and algal species are often placed in one of three major groups based upon plastidial characters; the green plant lineage (including green algae and land plants), the rhodophyte algae and the chromophyte algae. While chloroplasts from the green lineage may represent a monophyletic assemblage arising from a cyanobacterium-like ancestor, recent studies indicate that plastids of the

The nucleotide sequence data reported will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X61179.

rhodophytes and chromophytes have characteristics quite distinct from those of green plants [1, 2, 4, 8, 39], and that multiple endosymbiotic events may be necessary to explain their evolution [35]. In an effort to understand the series of events leading to the establishment of plastids in algae outside the green lineage, we have undertaken the characterization of the chloroplast genome of the brown alga *Pylaiella littoralis* (L.) Kjellm.

P. littoralis is a marine macroalga of the family Ectocarpaceae, division Phaeophyta. Its chloroplast genome is composed of two distinct circular molecules which have been visualized by electron microscopy and mapped by restriction endonuclease digestion and by hybridization to chloroplast gene probes [6, 22]. The larger of the two molecules (133 kb) is typical of many chloroplast genomes in that it carries two transcribed rRNA operons on inverted repeats. The smaller molecule (57.8 kb) carries an untranscribed 16S rRNA pseudogene [25] as well as sequences homologous to parts of the 23S rRNA gene. The organization of the transcribed rRNA operons has been studied [23] and is very similar to that of known eubacterial and cyanobacterial rRNA operons. The 16S rRNA gene is found at the 5′ end of the operon followed by $tRNA^{Ile}$ and $tRNA^{Ala}$, which are followed in turn by the 23S and 5S rRNA genes. There are no introns present within the genes, and the 3′ end of the 23S rRNA does not appear to be processed to yield a 4.5S rRNA species [23]. Sequence data have been published for the 16S rRNA gene [25], the spacer region between the 16S and 23S rRNA genes [26], the 4.5S rRNA-like region of the 23S rRNA gene, the spacer region between the 23S and 5S rRNA genes [23], and the 5S rRNA gene [35]. With the addition of the remaining sequence of the 23S rRNA gene, reported herein, the entire sequence of the ribosomal RNA operon of *P. littoralis* is now known. The thorough characterization of this operon not only affords the opportunity to study the phylogenetic position of the *P. littoralis* plastid, but also to compare the respective relations of the purple eubacterial and cyanobacterial rRNA species in reconstructed evolutionary relationships.

Materials and methods

Sequence analyses

Pylaiella chloroplast DNA was isolated and purified as previously described [6]. Cloned *Eco* RI restriction fragments containing the 23S rRNA gene [22] were sequenced entirely in both directions using a dideoxy sequencing kit (Pharmacia) with synthetic oligonucleotide primers (MedProbe S.A., Oslo, Norway).

Analysis of the cloned DNAs indicated that an *Eco* RI fragment encompassing ca. 700 bp of the 23S rRNA gene was not represented in the clone bank. This fragment was isolated from total chloroplast DNA via amplification by polymerase chain reaction (PCR) [32] using oligonucleotide primers complementary to the 5′ and 3′ proximal regions of the 23S rRNA gene (Fig. 1). The amplification conditions were as described by Sambrook *et al.* [34] using a hot start (reactions were

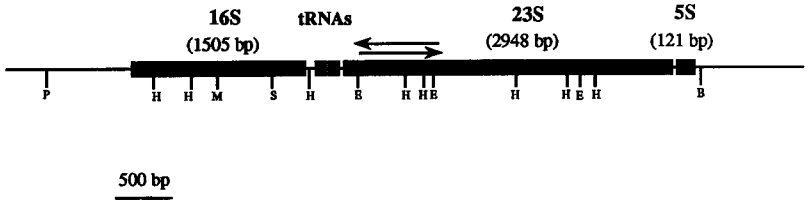

Fig. 1. Genetic organization of the sequenced plastidial rDNA operon of the brown alga *Pylaiella littoralis*. Genes denoted by solid rectangles are from left to right: the 16S rRNA, $tRNA^{ala}$, $tRNA^{ile}$, 23S rRNA and 5S rRNA genes. The 23S region delimited by arrows was sequenced from amplification products as indicated in Materials and methods. B = *Bgl* II, E = *Eco* RI, H = *Hin*d III, M = *Mlu* I, S = *Sma* I, P = *Pst* I. The entire nucleotide sequence between the *Pst* I and *Bgl* II endonuclease restriction sites is available in the EMBL Data library under the accession number X61179.

heated to 94 °C for 5 min prior to the addition of *Taq* I polymerase) followed by 25 thermal cycles (1 cycle of 5 min at 94 °C, 2 min at 46 °C, 5 min at 72 °C followed by 23 cycles of 1 min at 94 °C, 2 min at 46 °C, 5 min at 72 °C, and finishing with 1 cycle of 1 min at 94 °C, 2 min at 46 °C, 10 min at 72 °C). Specific amplification products were recovered from as little as 0.2 ng total plastidial DNA. The amplification products were cut with *Eco* RI, and ligated to M13mp19 cut with *Eco* RI and treated with calf intestinal phosphatase (Boehringer-Mannheim). Insert sequence from individual recombinant phage was compared to sequence from the analogous region of the *Anacystis nidulans* 23S rRNA gene [7] to verify the identity of the inserts. Phage DNAs were pooled from 19 independent clones with the same insert orientation, and the entire sequence of the insert was determined by dideoxy sequencing as described above.

Secondary structure model and phylogenetic analyses

The secondary structure model for the *P. littoralis* chloroplast 23S rRNA (Fig. 2) was constructed by comparison to previously published models [30], and according to modifications established by Guttel and Fox [15]. Fifteen chloroplast and prokaryotic 23S rRNA sequences were manually aligned, using the secondary structure models to determine the position of analogous regions within each molecule. Bases which occurred in hypervariable regions or which could not be aligned with confidence were removed, yielding an alignment of 2711 positions for comparison in each sequence. The alignment is not shown, but is available upon request. An estimation of sequence divergence was made for all possible sequence pairs using the Kimura two-parameter model [19], and the resulting distance matrix was analysed by the Neighbor Joining method [33]. The resulting phylogeny was tested by bootstrap analysis (DNABOOT of PHYLIP v3.4) [11] in order to determine confidence limits for the tree topology presented (Fig. 3).

Results and discussion

Secondary structure model

The entire nucleotide sequence of the *P. littoralis* chloroplast 23S rRNA gene (2948 bp) has been determined. The proposed secondary structure for the 23S rRNA, derived from the gene sequence, is shown in Fig. 3. The secondary structure model shown here is very similar to that of the cyanobacterium *Anacystis nidulans* [7, 15]. Where the two sequences differ, the integrity of the proposed helical regions is maintained by complementary base changes downstream. Regions of the model that are notably different from that of *A. nidulans* and the known plastidial models are marked in Fig. 2 (as A, B, C...) and discussed below.

The small stem and loop structure at position 530 in the *Pylaiella* model (A in Fig. 2) has only two base pairs in the stem. All other known 23S rRNAs have a 4 bp stem at the analogous position.

The chloroplast 23S rRNAs of land plants and euglenoid algae have inserts of 20 nt and 3 nt, respectively, at position 981 (B in Fig. 3). This feature is absent in both the *Pylaiella* and *Anacystis* molecules.

The stem and loop structure at position 1220 of the *P. littoralis* 23S rRNA model (C in Fig. 2) is enlarged by 17 to 19 nt relative to most other plastids and *A. nidulans*, or 11 to 18 nt when compared to known prokaryotic and plant mitochondrial sequences. By contrast, a very large insert is found in the same position in the 23S rRNA of *Chlorella ellipsoides* [41]. In the *Pylaiella* molecule, the additional bases appear to come from a duplication of part of the stem and loop inserted in the loop in inverse position (left to right).

In the plastidial 23S rRNAs of all known land plants and *Chlorella*, there is an insert at position 1537 or 1558 (D in Fig. 2) which forms an additional loop structure. The loop does not exist in *Pylaiella*, nor in the 23S rRNAs of free-living prokaryotes.

The stem and loop structure found at position 1763 (E in Fig. 2) has only a 3 bp helix. This is

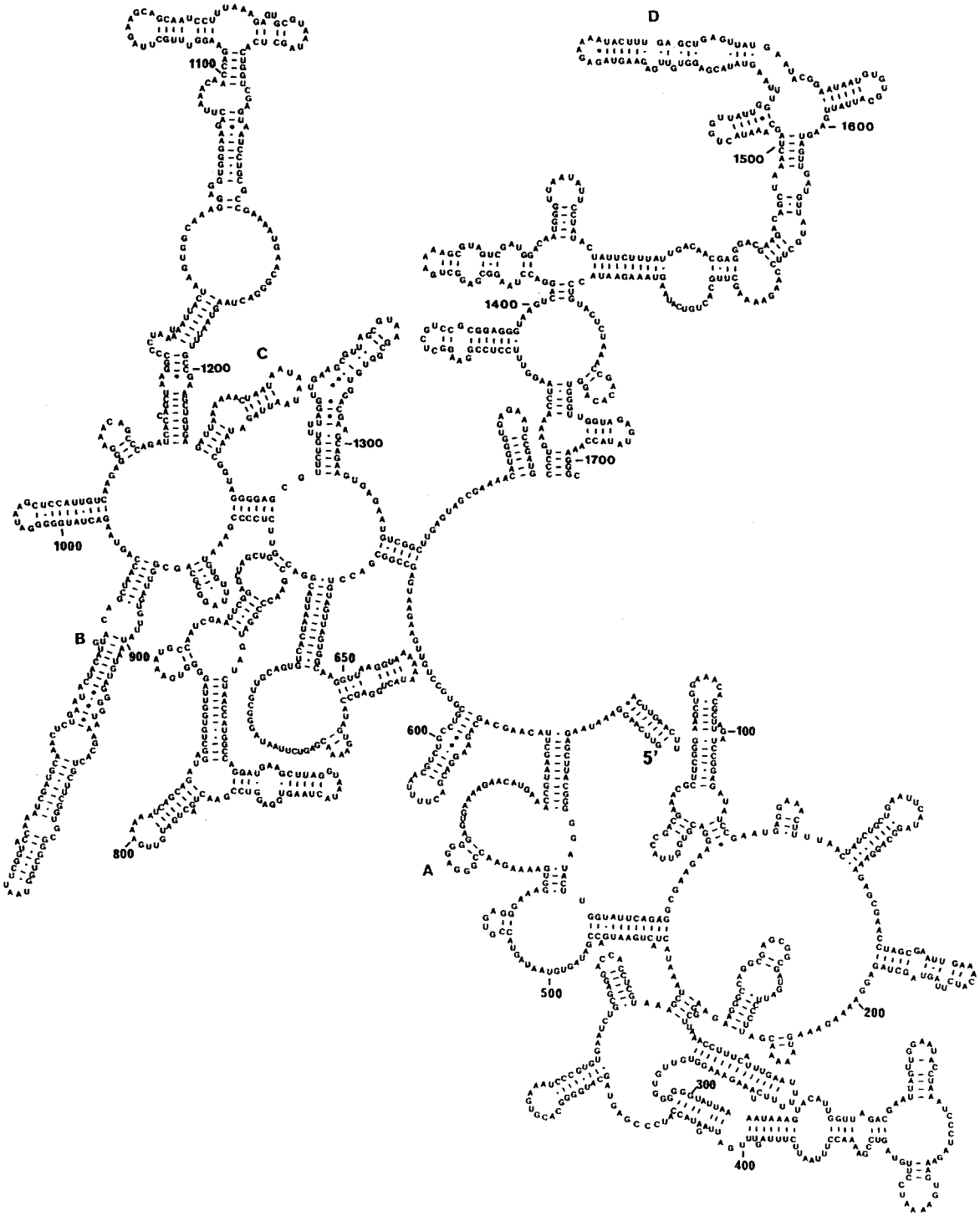

Fig. 2. Proposed secondary structural model of the *P. littoralis* 23S rRNA constructed according to [15]. 'A' to 'H' indicate special features of this model discussed in the text.

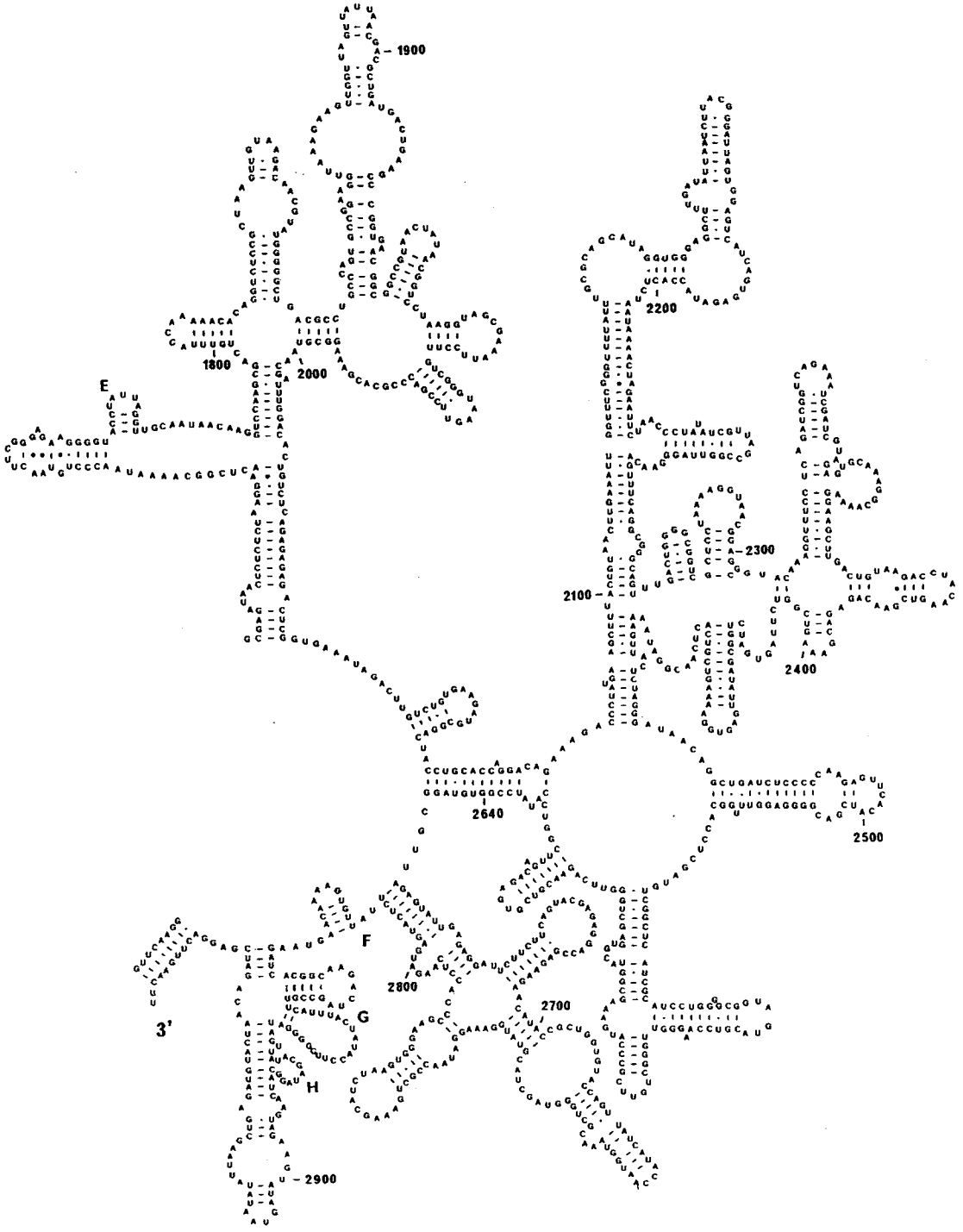

Fig. 2. (continued).

smaller than the analogous structure in any other known 23S rRNA model.

A short stem and loop structure is found at position 2812 (F in Fig. 2) which is similar to those found in the prokaryotes (including cyanobacteria) and the green algae. In land plants a

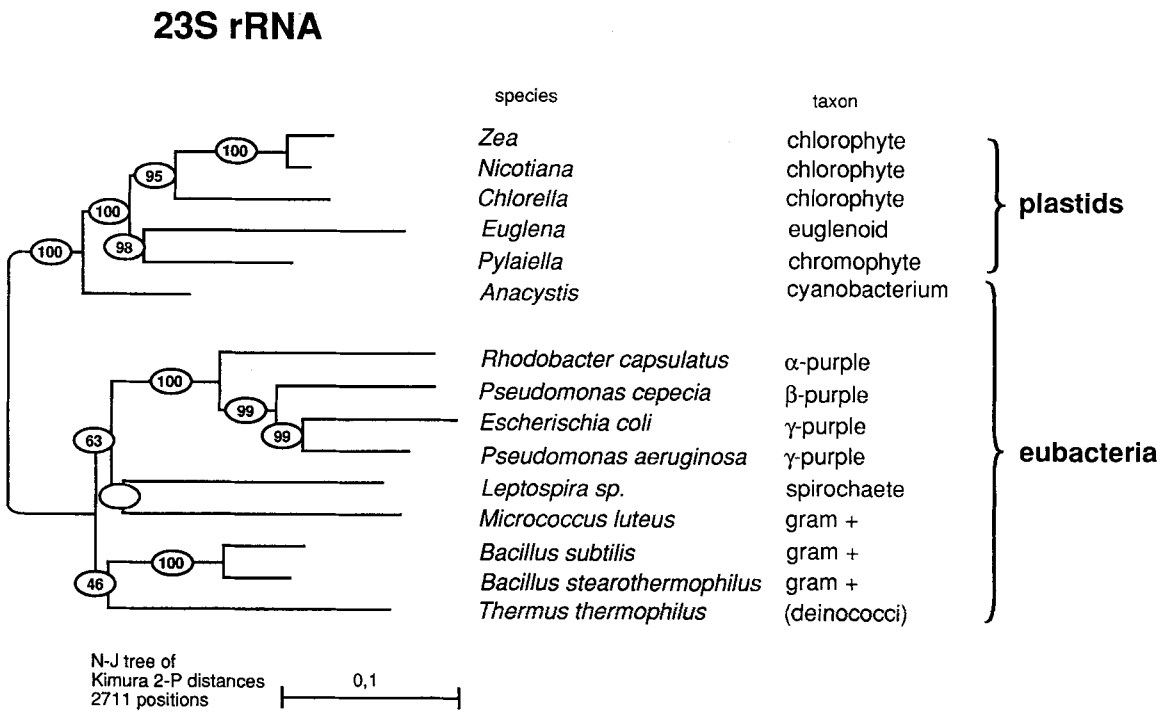

Fig. 3. Neighbor-Joining tree [33] for values of divergence between 23S rRNA sequences as measured by the Kimura two parater method [19]. The scale bar represents 0.1 mutations per site (Kumura 2-P), branch lengths (horizontal) are drawn to scale. Numerical values at nodes represent the percentage of instances in which the species drawn to the right on a given branch occurred to the right on that branch in a set of 100 trees generated from a bootstrap analysis [11]. Species are: *Zea mays* [10], *Nicotiana tabacum* [36], *Chlorella ellipsoidea* [41], *Euglena gracilis* [42], *Pylaiella littoralis* (this work), *Anacystis nidulans* [7], *Rhodobacter capsulatus* [16], *Pseudomonas cepacia* [17], *Escherichia coli* [5], *Pseudomonas aeruginosa* [37], *Leptospira interrogans* [12], *Micrococcus luteus* [31], *Bacillus subtilis* [14], *Bacillus stearothermophilus* [20], *Thermus thermophilus* [18]. The leftmost branch of the tree was simply arbitrarily 'bent' to permit display of species names in a convenient manner.

relatively large spacer region occurs at this position which is spliced out to yield the 23S and 4.5S rRNAs.

The 4.5S rRNA-like region of *Pylaiella* has been previously described [23]. An alignment of homologous RNAs from prokaryotes and chloroplasts indicates an insertion at position 2851 that is 1 nt in *Euglena*, 2 nt in monocots, 8 nt in dicots and 18 nt in *Pylaiella* (G in Fig. 2) relative to the prokaryotic rRNAs. A second insertion (H in Fig. 2) at position 2876 is unique to *Pylaiella* among known sequences. Both inserts form loops in the 4.5S rRNA-like region of the *P. littoralis* 23S rRNA that are not found in other plastidial or prokaryotic rRNAs.

The phylogeny of chloroplast rRNAs

Figure 3 indicates the phylogenetic position of the *P. littoralis* plastid among several chloroplasts and eubacteria based on 2711 positions of the 23S rRNA gene. The most striking aspects of the tree are the clear separation of the cyanobacterial lineage from the remainder of the prokaryotes, and the clustering of all plastidial sequences within the cyanobacterial group. Similar gross topologies have been found for phylogenetic trees based on 5S rRNA [35], and 16S rRNA [9, 24, 27]. Markowicz and Loiseaux-de Goër [27] found two plastidial radiations within the cyanobacterial lineage; one giving rise to the chloroplasts of

green plants and green algae, and the other comprising the plastids of the rhodophyte, chromophyte and euglenoid algae. These lines appeared to have arisen independently from the cyanobacterial line, although the resolution afforded by the 16S rRNA sequences was not enough to place the branching orders with statistical significance. A cladistic analysis of 5S rRNA sequences [35] likewise indicated that the green lineage and the red-brown lineage arose independently from within the cyanobacterial radiation, but placed *Euglena* in a clade containing green algae and land plants. Figure 3 shows two plastid lines, one including green plants and a green alga, and the other including a chromophyte and a euglenoid alga (unfortunately, no 23S rRNA sequences from rhodophyte plastids were available for inclusion in the present study). Thus the 23S rRNA analysis supports that based on 16S rRNA regarding the position of the euglenoid algae. Clearly, the association of the *Pylaiella* and *Euglena* sequences is significant as these two sequences clustered together in 98 of 100 bootstrap replicates [11], as is the placement of the chromophyte-euglenoïd branch within a single plastid group (100 of 100 bootstrap replicates). This indicates that plastids of all types could, but do not necessarily, share a common cyanobacterial ancestor. In fact, the topology shown in Fig. 3 cannot be interpreted to support either monophylesis or polyphylesis of chloroplasts without considering the timing of endosymbiosis. A single endosymbiotic event occurring shortly after divergence from the cyanobacterial line would be consistent with monophylesis, while a relatively late establishment of endosymbiosis (sometime after divergence of the green and brown lines) would support polyphylesis.

One must therefore look at the studies of plastidial phylogeny based on other genes in order to obtain a better resolution of timing of endosymbiosis. Phylogenetic studies dealing with the large and small subunits of ribulose bisphosphate carboxylase/oxygenase (Rubisco) contrast sharply with the rRNA-based data [1, 2, 4, 8, 21, 28, 38, 39, 40]. It was shown that while the Rubisco of *Euglena* is closely related to the Rubiscos of green algae and land plants, those of chromophytes and rhodophytes cluster with those of α (type I) and β-purple eubacteria [28]. Similarly, a phylogenetic study based on *psaB* amino acid sequences [3] places the *E. gracilis* gene much closer to homologous genes of green plants than to that of *Pylaiella*. These data have been interpreted as indicating that the plastids of the rhodophyte and chromophyte algae are of a composite origin [1, 2, 3, 4, 29], i.e. that these plastids have characteristics gained by horizontal as well as vertical inheritance (see also [28] for alternate hypotheses), and that those of euglenoids could also be of composite origin [27]. It is to be noted that euglenoid plastids are surrounded by an extra membrane (CER) which sets them apart from the green algae and land plants and has been interpreted as a sign of secondary endosymbiosis [13]. That the composite genome of the red-chromophyte lineage differs from that of the *Euglena* lineage argues that acquisition of these genotypes occurred after the divergence of their plastid lineages and tends to support a polyphyletic theory of plastidial evolution. Unfortunately, these studies do not truly resolve the timing of endosymbiosis, since gene transfer inside the host cell cannot be ruled out.

In conclusion, the secondary structure model presented here is the first such model of a 23S rRNA from a chromophyte chloroplast. The overall structure is more similar to that of the 23S rRNA from the cyanobacterium *Anacystis nidulans* than to those of the purple eubacteria, gram positive bacteria, or green algae and land plants. There are a number of features of the molecule that are unique and may prove to be characteristic of red and chromophyte plastids as more sequences become available. Similarly, the phylogeny of plastids based on 23S rRNA sequences requires the addition of other rhodophyte, chromophyte and euglenoid sequences to the database. Too few 23S rRNA sequences are known to date to fully describe the history of plastidial evolution from these data.

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